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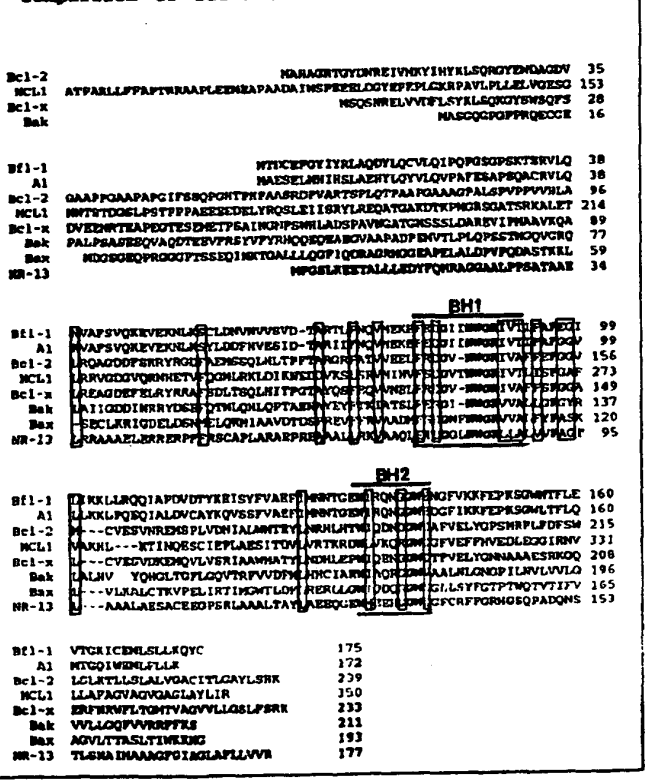
(54) Title: APOPTOSIS REGULATING GENE

(57) Abstract

A new Bcl-2 related gene "Bfl-1", a polypeptide encoded by said gene, and a plasmid and a transformant comprising said gene are disclosed. The gene can be used to detect cancer.



APOPTOSIS REGULATING GENE

BACKGROUND OF THE INVENTION

1. FIELD OF THE INVENTION

The present invention is related to a new apoptosis regulating gene, a plasmid containing the same, and a peptide encoded by the same.

Several publications are referenced in this application. Full citation to these references is found at the end of the specification immediately preceding the SEQUENCE LISTING or where the publication is mentioned; and each of these publications is hereby incorporated herein by reference.

2. BACKGROUND OF THE INVENTION

Living cells are programmed to be spontaneously died when they are useless. Such a programmed cell death is called "Apoptosis" and has widely attracted attentions in cell physiological fields.

Apoptosis plays an important role in many physiological processes, such as embryonic development, deletion of self-reactive T-cells (Williams, 1991; Williams and Smith, 1993), and in many diseases such as cancer or neurodegenerative disorders. Apoptosis also plays a critical role in maintaining homeostasis in many adult tissues. It is widely accepted that cell death and cell proliferation are precisely balanced to maintain the proper types of cells or tissues, and disruption of this balance can result in several

carcinogenesis.

Bcl-2 is homologous to the C. elegans ced-9 gene, an apoptosis-blocking gene (Hengartner and Horvitz, 1994), and is abundantly expressed in follicular lymphoma that is resulted from the t(14;18) chromosomal translocation (Tsujimoto *et al.*, 1985). It has been known that deaths of a variety of cell types can be prevented by Bcl-2 overexpression, although not all forms of cell death are inhibited (Williams, 1991). Thymocyte overexpressing Bcl-2 were resistant to the induction of apoptosis by glucocorticoid, radiation or anti-CD3 treatments (Sentman *et al.*, 1991; Strasser *et al.*, 1991). Overexpression of Bcl-2 in B cell compartments increases the number of mature resting B cells (Strasser *et al.*, 1991, due to extended cell survival rather than increases proliferation. The action mechanism of the Bcl-2 is not clear yet. Recently, Bcl-2 has been reported to protect apoptosis independent of the inhibition of reactive oxygen speciés (Jacobson and Raff, 1995; Shimize *et al.*, 1995), which is contradictory to the previous results (Hockenbery *et al.*, 1993).

Several genes with Bcl-2 related sequences have been reported. Bax, 21 kDa protein, was known to have 21% homology to Bcl-2, and inhibits the function of Bcl-2, perhaps by forming Bcl-2-Bax complex (Oltvai *et al.*, 1993). Bcl-x was reported to have a high-level homology to Bcl-2 and like Bcl-2 prevents apoptotic cell death in IL3-dependent cells following growth factor deprivation (Boise et al., 1993). Bak was

that oncogenesis can be induced by another mechanism, that is, failure of appropriate cell death rather than activation of cellular proliferation.

Under the circumstance that the mechanism of apoptosis at the molecular level is not understood, a finding of a new gene involved in the regulation of apoptosis can accelerate or help an understanding of the mechanism. The new gene can be used to develop a useful diagnostic agent or cancer therapy.

SUMMARY OF THE INVENTION

The present invention pertains to a new Bcl-2 related genes.

One object of the present invention is to provide Bfl-1 gene, a Bcl-2 related gene from a human fetal liver, comprising bases in SEQ ID NO:1 or equivalents thereof.

Other object of the present invention is to provide a plasmid containing the Bfl-1 gene.

Another object of the present invention is to provide a transformant bearing the plasmid.

Still another object of the present invention is to provide a polypeptide comprising amino acids 1 - 175 of SEQ ID NO:2.

BRIEF DESCRIPTION OF THE DRAWINGS

cancer tissues. Eight sets of stomach tissues were obtained from eight different stomach cancer patients. Each set consisted of a normal stomach, tumor tissue and metastatic tumor nodule. Photograph of the corresponding ethidium bromide-stained gel is shown below each autoradiogram. M: metastatic nodule, T:tumor tissue, N: normal stomach tissue.

DETAILED DESCRIPTION OF THE INVENTION

The new gene according to the present invention was originally isolated from a human fetal liver at 22 week of gestation and identified by computer analysis of expressed sequenced tag (EST) databases constructed by single-pass sequencing of random cDNA clones (Choi *et al.*, 1995).

A directional cDNA library was constructed from total RNA from a 22 week old human fetal liver, and used for its amplification by using competent cells. The DNAs were sequenced and each clone was identified. Among the sequenced clones, a clone showing a homology with murine A1 gene was selected and designated as "f1-383d." The f1-383d clone proved to be a new member of the Bcl-2 related gene family, and the new Bcl-2 related gene was named "Bf1-1" ("Bcl-2 related gene expressed in fetal liver").

Bf1-1 gene has a homology with Bcl-2, especially within the BH1 and BH2 domains. Bcl-2 has been shown to prevent apoptotic cell death in cultured cells

by forming heterodimers, Bcl-2-Bax complexes (Oltvai *et al.*, 1993).

Accordingly, the fact that Blf-1 contains BH1 and BH2 domains strongly suggests that the gene may also be involved in the regulation of apoptosis.

The Bfl-1 gene is highly expressed in bone marrow and present in hemopoietic lineages such as Raji and HL60 and in some normal adult tissues, including lung, spleen, and esophagus. The expression patterns of other Bcl-2 related genes are very distinctive. Bcl-2 is expressed in bone marrow progenitors or long-lived cells in hormonally responsive epithelia that undergo cycles of hyperplasis and in neurons of the peripheral neuronal system (Veis *et al.*, 1993). Bcl-x is highly expressed in the thymus and the central nervous system (Boise *et al.*, 1993). Bax expression was not lymphoid restricted but was widely expressed in a variety of tissues, including lung, stomach, kidney, thymus, bone marrow and spleen (Oltvai *et al.*, 1993). Like Bax, Bak is ubiquitously expressed. A1 is a hemopoietic-specific gene expressed in several hemopoietic cell lineages (Lin *et al.*, 1993). It is notable that the expression of apoptosis-accelerating genes such as Bax and Bak is widespread in different tissues, whereas the expression of apoptosis-blocking genes such as Bcl-2, Bcl-x and A1 is restricted in some tissues, suggesting that the activity of apoptosis-acceleratng genes may be regulated by death inhibitory genes.

Interestingly, the expression of Bfl-1 appears to

al., 1994).

The present invention also provides equivalent DNA constructs that encode various additions or substitutions of amino acid residues or sequences, or deletions of terminal or internal residues or sequences not needed for biological activity of the polypeptide comprising the amino acids 1 - 175 of SEQ ID NO:2.

Nucleic acid sequences within the scope of the invention include isolated DNA and RNA sequences that hybridize to the cDNA nucleotide sequences disclosed herein under conditions of moderate or severe stringency, which encode the polypeptide comprising the amino acids 1 - 175 of SEQ ID NO:2. Conditions of moderate stringency, as defined by Sambrook *et al.*, Molecular Cloning : A Laboratory Manual, 2nd ed, Vol. 1, pp.1.101-104, Cold Spring Harbor Laboratory Press, (1989), include use of a prewashing solution of 5 X SSC, 1.0 mM EDTA (pH 8.0) and hybridization conditions of about 55°C, 5 X SSC, overnight. Conditions of severe stringency include higher temperatures of hybridization and washing. The skilled artisan will recognize that the temperature and wash solution salt concentration may be adjusted as necessary according to factors such as the length of the probe.

Due to the known degeneracy of the genetic code wherein more than one codon can encode the same amino acid, a DNA sequence may vary from that shown in SEQ ID NO:1 and still encode the polypeptide having the amino acid sequence of SEQ ID NO:2.

reaction, samples were run in a 4.5% polyacrylamide gel. The gel was soaked in a solution consisting of 10% of methanol and 10% of acetic acid for 15 to 30 min, dried, and exposed to an X-ray film at room temperature for 12 to 14 hours.

Comparison between the generated sequences and public databases (Genbank, SWISS-PORT and PIR) was performed using BLAST program. The multiple sequence alignment was performed using the software package IG suite (IntelliGenetics Co, Mountain View, CA), installed on a SUN SPARC Staion 2 computer (SUN Microsystems, Inc, Mountain View, CA).

3. Northern analysis

Total RNA of tissues and cell lines was isolated using guanidine thiocyanate protocol (Sambrook *et al.*, 1989). About 20-30 μg of total RNA was loaded per lane of 1% denaturing formaldehyde agarose gel and run at 40V for 16 hours. The RNA was transferred to a nylon membrane (Schleicher and Schuell) using 10 X SSC. Radioactivity labeled probes (2 x 10^5 c.p.m. per ml) were hybridized to the blots at 65°C in a buffer as described by Church and Gilbert (1984). After 18 h hybridization, the filters were washed in a buffer containing 0.1 X SSC, 0.1% SDS at 65°C for 15 min. The autoradiogram was taken for 1-7 days (Kang *et al.*, 1994).

Results

1. Identification of a novel gene related to Bcl-2

"-4" is given when the amino acids compared are the different from each other. Therefore, the higher the score, the more homologous the amino acid sequences. The "p-value" also is a computerized value and indicates the possibility of an accidental match. Accordingly, the lower the value, the more specific the match.

The results of amino acid homology evaluation reveals that the Bfl-1 products shows the highest homology with the polypeptide L16462, an A1 gene product.

The detailed comparison of amino acids of the Bfl-1 and A1 products are shown in FIG. 2. As can be seen from FIG. 2, Bfl-1 gene shows similarity throughout the partially sequenced bases from its first amino acid, Met. The size of cDNA insert is about 750bp, which is similar to that of A1 transcript.

The new Bcl-2 related gene according to the present invention is now named "Bfl-1 (Bcl-2 related gene expressed in fetal liver)."

The A1 gene is a Bcl-2 related gene in the mouse and is known as a hemopoietic specific dearly response gene whose transcription is rapidly and transiently induced by GM-CSF in murine bone marrow-derived macrophage (Lin *et al.*, 1993). Since the Bcl-2 related genes play an important role in the regulation of apoptosis, the present inventors have determined the full DNA sequence of the novel cDNA gene and deduced the amino acid sequence of a potential open reading frame (SEQ ID NO:1). Bfl-1 is consisted of 734 bp and

the BH1 and BH2 domains, which have been known to be important for Bcl-2 function, indicating that the human cDNA clone f1-383d represents a new member of the Bcl-2 related gene family.

2. The Bfl-1 gene is abundantly expressed in bone marrow and at a low level in some other tissues

To examine the expression pattern of Bfl-1, northern blot analysis was performed on various human tissues and cell lines (FIG. 5). Because Bfl-1 was initially identified from a human fetal liver at 22 weeks of gestation, which consists of hepatic and hemopoietic cells, the present inventors performed northern analysis of fetal liver, hemopoietic lineage cells such as HL60 and Raji, and primary acute lymphocyte leukemia (ALL) cells. The results are shown in FIG. 4. Bfl-1 was highly expressed in bone marrow, but not detected in fetal liver, indicating that the level of Bfl-1 expression is not high in the fetal liver (FIG. 4A). The Raji cell line derived from Burkitt's lymphoma expressed Bfl-1, whereas the other cell lines did not express Bfl-1, or did very little, if any. On northern analysis with various normal adult tissues, Bfl-1 message was detected at low levels in lung, spleen, and esophagus (FIG. 4B). No Bfl-1 message was detected in several other nonhemopoietic tissues including heart, testis, thyroid, cerebellum and cerebrum.

3. The expression of Bfl-1 is activated in stomach

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SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:
(A) NAME: Korea Green Cross Corporation
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(D) STATE: Kyoungbuk
(E) COUNTRY: Republic of Korea
(F) POSTAL CODE (ZIP): 790-330

(ii) TITLE OF INVENTION: Apoptosis Regulating Gene

(iii) NUMBER OF SEQUENCES: 2

(iv) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0,
Version #1.30 (EPO)

(v) CURRENT APPLICATION DATA:
APPLICATION NUMBER: To be assigned
(vi) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: KR 1995-6266
(B) FILING DATE: 24-MAR-1995

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 755 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Homo sapiens
(D) DEVELOPMENTAL STAGE: Fetus
(F) TISSUE TYPE: Liver

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 175 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

Met Thr Asp Cys Glu Phe Gly Tyr Ile Tyr Arg Leu Ala Gln Asp Tyr
1 5 10 15

Leu Gln Cys Val Leu Gln Ile Pro Gln Pro Gly Ser Gly Pro Ser Lys
20 25 30

Thr Ser Arg Val Leu Gln Asn Val Ala Phe Ser Val Gln Lys Glu Val
35 40 45

Glu Lys Asn Leu Lys Ser Cys Leu Asp Asn Val Asn Val Val Ser Val
50 55 60

Asp Thr Ala Arg Thr Leu Phe Asn Gln Val Met Glu Lys Glu Phe Glu
65 70 75 80

Asp Gly Ile Ile Asn Trp Gly Arg Ile Val Thr Ile Phe Ala Phe Glu
85 90 95

Gly Ile Leu Ile Lys Lys Leu Leu Arg Gln Gln Ile Ala Pro Asp Val
100 105 110

Asp Thr Tyr Lys Glu Ile Ser Tyr Phe Val Ala Glu Phe Ile Met Asn
115 120 125

Asn Thr Gly Glu Trp Ile Arg Gln Asn Gly Gly Trp Glu Asn Gly Phe
130 135 140

Val Lys Lys Phe Glu Pro Lys Ser Gly Trp Met Thr Phe Leu Glu Val
145 150 155 160

Thr Gly Lys Ile Cys Glu Met Leu Ser Leu Leu Lys Gln Tyr Cys
165 170 175

9, wherein said cancer is stomach cancer.

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FIG. 2

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Bfl-1   MTDCEFGYIYRLAQDYLQCVLQIPQPGSGPSKTSRVLQNVAFSVQKEVEKNLKSCLDNVNV 61
        M + E  +I+ LA+ YLQ VLQ+P   S PS+  RVLQ VAFSVQKEVEKNLKS LD+ +V
A1      MAESELMIIIHSLAEHYLQYVLQVPAFESAPSQACRVLQRVAFSVQKEVEKNLKSYLDDFHV 61

Bfl-1   VSVDTARTLFNQVMEKEFEDGIINWGRIVTIFAFEGILIKKLLRQQIAPDVDTYKEISYFV 118
         S+DTAR +FNQVMEKEFEDGIINWGRIVTIFAF G L+KKL ++QIA DV  YK++S FV
A1      ESIDTARIIFNQVMEKEFEDGIINWGRIVTIFAFGGVLLKKLPQEQIALDVCAYKQVSSFV 122

Bfl-1   AEFIMNNTGEWIRQNGGWENGFVKKFEPKSGWMTFLEVTGKICEMLSLLKQYC 175
        AEFIMNNTGEWIRQNGGWE+GF+KKFEPKSGW+TFL++TG+I EML LLK
A1      AEFIMNNTGEWIRQNGGWEDGFIKKFEPKSGWLTFLQMTGQIWEMLFLLK    172
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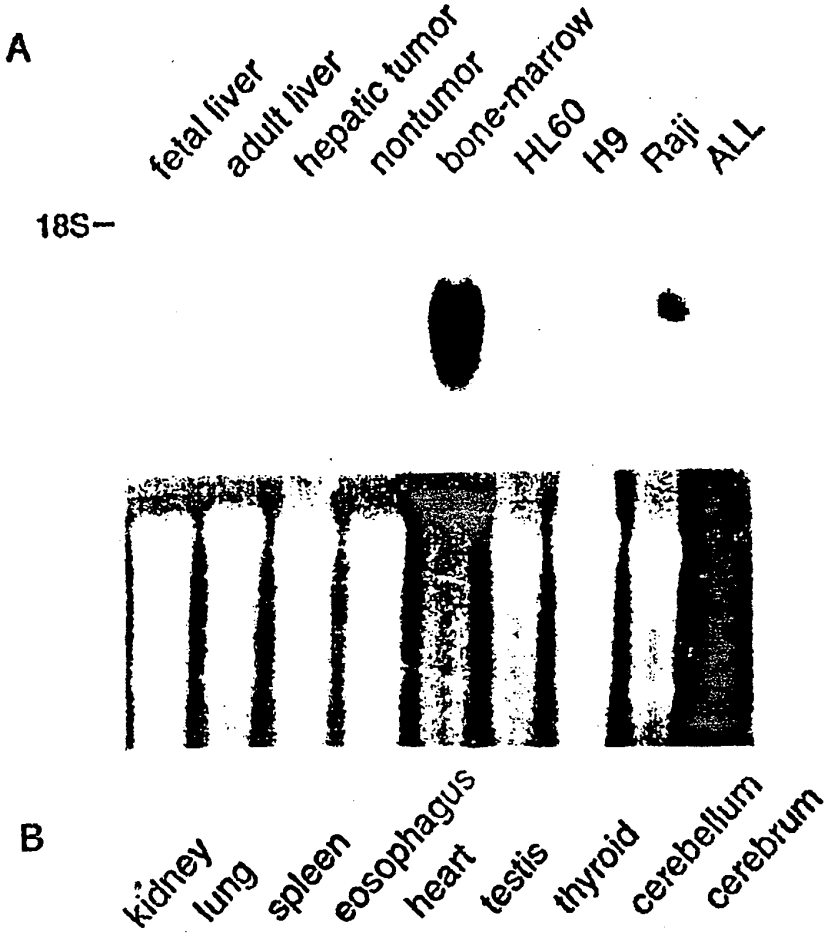
FIG. 4

Northern blot analysis of Bfl-1 gene expression in several human tissues and cell lines



INTERNATIONAL SEARCH REPORT

International application No.
PCT/KR 96/00040

A. CLASSIFICATION OF SUBJECT MATTER

IPC[6]: C 12 N 15/12; C 12 Q 1/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC[6]: C 12 N 15/12; C 12 Q 1/68

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WPI, CAS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 93/20 200 A1 (IMPERIAL CANCER RESEARCH TECHNOLOGY LIMITED) 14 October 1993 (14.10.93), claim 26; pages 75-78.	1-10
A	THE JOURNAL OF IMMUNOLOGY, Vol.151, No.4, 15 August 1993 (15.08.93), Baltimore, USA E.Y.LIN et al.:"Characterization of A1, a Novel Hemopoietic - Specific Early - Response Gene with Sequence Similarity to bcl-2", pages 1979-1988; page 1981, fig.1.	1-10
A	The EMBO Journal, Vol.14, No.5, 01 March 1995 (01.03.95), Oxford (GB), G. GILLET et al.:"A BCL-2-related gene is activated in avian cells transformed by the Rous sarcoma virus", pages 1372-1381; page 1376.	1-10
A	CELL, Vol.76, No.4, 25 February 1994 (25.02.94), Cambridge (Mass., USA), M.O. HENGARTNER et al.: C.elegans Cell Survival Gene ced-9 Encodes a Functional Homolog of the Mammalian Proto-Oncogene bcl-2", pages 665-676; page 670, fig.7.	1-10

☐ Further documents are listed in the continuation of Box C. ☒ See patent family annex.

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
12 June 1996 (12.06.96)	19 June 1996 (19.06.96)

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